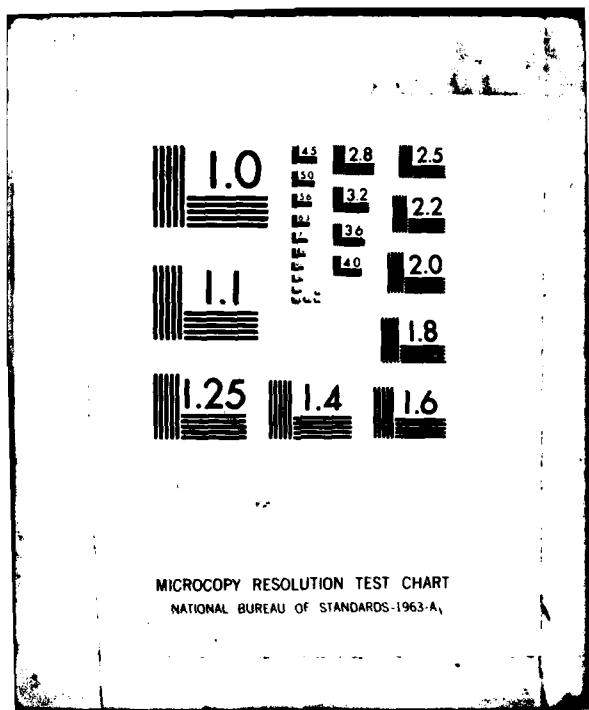


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OFFICE OF NAVAL RESEARCH

Contract N00014-75-C-0634

Work Unit No. NR 204-015

FINAL REPORT

The Physiological Bases for Microbial
Barotolerance

by

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13 November 1981



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TABLE OF CONTENTS:

A. Summary of All Research Accomplished;.....	3
B. Index of Annual Reports Issued Under the Contract;.....	10
C. Index of Publications Issued Under the Contract;.....	11
D. Conclusions Drawn on the Basis of the Results;.....	11
E. A List of Major Accomplishments.....	12
DD Form 1473.....	14
Distribution List.....	16

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A. Summary of All Research Accomplished

It seems appropriate that the main body of this summary should be the abstracts of the annual technical reports submitted during the contract period. However, a few initial comments will help to put these abstracts into the proper context.

> The long-range goals of our barophysiology program are to define the bases for differences in barotolerance among microorganisms in terms of the pressure sensitivities of specific biochemical or physiological processes and to explore ways to use pressure advantageously in basic and applied microbiology. These goals have been expanded in recent years to include acquisition of knowledge of the mechanisms by which high-pressure gases affect cell growth. The abstracts below reflect these two main themes.

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Abstract of 31 December 1975 - The inhibition of streptococcal growth by hydrostatic pressure was found to be the result of an increased demand for adenosine triphosphate under pressure coupled with a somewhat diminished supply. The increased demand seemed to be due to pressure stimulation of the membrane adenosine triphosphatase of the bacterium.

It was found also that pressure markedly upsets electrolyte balances in organisms such as Streptococcus faecalis but not in organisms such as Escherichia coli or Bacillus licheniformis.

Data are presented in this report to suggest that one atmosphere is not the optimal growth pressure for many bacteria, but that growth at 100 atmospheres and a temperature slightly above the optimum is faster and more extensive than is growth at any temperature at one atmosphere.

Data are presented also to show that bacterial growth under nonoptimal conditions is highly sensitive to pressure, and that at low temperatures in natural aquatic environments, pressures as low as 50 atmospheres can have major inhibitory effects on growth of mesophilic or psychrotrophic bacteria.

Finally, it was found that high-pressure oxygen stimulates synthesis and excretion of materials that absorb light of 260 nm wavelength and that the toxicity of oxygen may be related to derangements of nucleic acid metabolism.

Abstract of 31 March 1977 - Investigations during the past year of the effects of high-pressure narcotic gases have confirmed previous reports of inhibition of microbial growth and differentiation. Spore germination was found to be somewhat

more sensitive than was growth, and for example, germination of Bacillus cereus spores could be completely suppressed by only 10 atmospheres of nitrous oxide, compared with some 25 atmospheres required for 50% inhibition of growth of Streptococcus faecalis. High-pressure helium at 24 to 41 atmospheres was found to act oppositely in that it stimulated streptococcal growth and replication. This stimulation could be related to the finding that helium has negative narcotic potential for animals. Helium did act to enhance the narcotic potential of nitrous oxide and to increase oxygen toxicity, possibly because of a hydrostatic pressure effect rather than a specific gas effect. Oxygen appeared to act essentially as a narcotic gas in its inhibition of S. faecalis growth in a medium prepared with tryptone, glucose and yeast extract. The oxygen pressure required for 50% growth inhibition was about 25 atmospheres, approximately the pressure of nitrous oxide required for the same effect. However, long-term exposure to oxygen resulted in cell death, while similar exposure to nitrous oxide resulted in no killing. Moreover, supplementation of the complex growth medium, or a defined one, with phosphate buffer resulted in markedly enhanced oxygen sensitivity of the bacterium, and it was possible to obtain 50% growth inhibition with only about 15 atmospheres of oxygen.

Further studies of pressure-temperature interactions affecting microbial growth and physiology were undertaken. Efforts were made to enhance metabolite production by bacteria with increased hydrostatic pressure and to study molecular aspects of the interaction.

Abstract of 31 March 1978 - Attempts to relate growth inhibitory actions of anesthetic gases to their narcotic potencies revealed that the two are not well correlated. Instead, it was apparent that bacterial growth inhibition by the gases, is a unique class of actions that can be distinguished from general anesthetic or narcotic actions. The potency series determined for inhibition of growth of Escherichia coli B in complex medium at 22°C had N₂O and Xe > Kr > Ar, N₂ or He. The latter three gases had negative potencies in that they stimulated growth rather than inhibiting it. In the narcotic potency series only helium has a negative potency. Kr also could stimulate growth at pressures less than 20 atmospheres but it was inhibitory at higher pressures. The net conclusions here are that the potency series for growth inhibition differs from that for anesthesia and that different intracellular sites of action are likely.

Experiments with a series of bacteria indicated that there is a range of sensitivity to nitrous oxide and that nitrous oxide sensitivity is correlated with oxygen sensitivity. Staphylococcus aureus was more sensitive to the gases than was E. coli, and both were considerably more sensitive than was Streptococcus faecalis. For the streptococcus, which has only minimal capacity to metabolize oxygen and has protective peroxidase and superoxide dismutase enzymes, oxygen seemed to act mainly only as an anesthetic gas with a potency about equal to that of nitrous oxide.

The uniqueness of the growth inhibitory action was evident also in the effects of gas combinations on E. coli growth. Helium and anesthetic gases, including argon and nitrogen, were found to potentiate oxygen toxicity. For example, two atmospheres of oxygen or twenty atmospheres of nitrogen each alone had no effect or a slightly stimulatory effect on growth. When combined, they were highly inhibitory and almost completely suppressed growth. Combinations of anesthetic gases acted in a more than additive manner in inhibiting growth. Nitrogen was an exception in that it antagonized the action of, for example, nitrous oxide.

Preliminary experiments with the protozoan Tetrahymena pyriformis indicated that decompression after exposure to hyperbaric gases resulted in the formation of intracellular gas bubbles, which were never seen in decompressed bacterial cells. Therefore, to study the effects of high-pressure gases on Tetrahymena, it was necessary to construct a pressure chamber with optical windows so that growth could be monitored without the need for decompression and sample taking. Initial experimental results indicated that helium can reverse oxygen toxicity for Tetrahymena, which proved to be highly oxygen sensitive. Thus, helium behaved oppositely for eukaryotic cells than it did for prokaryotes. {Subsequent experiments indicated that the vacuoles formed in pressurized cultures of Tetrahymena were not gas filled vacuoles.}

During the next year, we shall continue work on the interactions of hydrostatic pressure, oxygen and anesthetic gases that affect growth with an aim to defining in more detail the basic responses of microorganisms, identifying major biochemical and physiological targets and comparing the responses of eukaryotic microorganisms with those of prokaryotes.

Abstract of 31 March 1979 - The results of experiments carried out during the past year gave further support to our view that the modifying actions of compressed gases on microbial growth cannot be considered narcotic actions but belong instead to a definably different class of action. A major component of this work involved the use of eukaryotic microorganisms.

Helium, nitrogen and argon at pressures as high as 60 atmospheres were found to have relatively little effect {slightly stimulatory} on growth of Escherichia coli, Saccharomyces cerevisiae or Tetrahymena thermophila (pyriformis). In the potency series for narcotic action, which has the order N_2O and $Xe > Kr > Ar > N_2 > Ne, H_2$ or He, there is a cross-over from negative potency to positive potency between He and Ne or H_2 . For growth effects, this cross-over from stimulatory or nil action to inhibitory action occurs at the level of Kr rather than He.

Although He, N_2 or Ar alone have only minor effects on growth, in combination with more potent gases such as N_2O they exert a dramatic potentiating action. The order of effectiveness for potentiation has the series Ar > N_2 > He. These gases also dramatically potentiate the toxic action of oxygen on growth. The potentiation can be demonstrated with prokaryotes {Escherichia coli} and also with eukaryotes which are phylogenetically related to plants {Saccharomyces cerevisiae} or to animals {Tetrahymena thermophila}.

During the past year, it has been possible to show that hydrostatic pressures of 100 or 200 atmospheres act to reverse the growth inhibitory effects of liquid narcotic agents of the aliphatic alcohol series. Higher pressures seem to enhance inhibition, and the data suggest multisite targets for growth modifying effects of narcotic agents.

Substantial advances have been made during the past year also in our study of the biological effects of hydrostatic pressure. In this work, we are concerned with pressure effects rather than with specific gas effects, and efforts are made to exclude gases from the test systems under study. By means of long-term cultivation of Streptococcus faecalis in agar stab cultures at pressures of 800 to 900 atmospheres, we have been able to select a variant bacterium with enhanced baro-tolerance. This variant is a stable mutant that occurs in small numbers in the parent population. Interestingly, it is not only baroduric but also aciduric. This combined resistance can be related to our previous findings that bacteria become sensitized under hydrostatic pressure to the growth inhibitory action of metabolic acids. The technique of prolonged culture in agar stabs under pressure with periodic transfer to new medium at one atmosphere offers a means for isolation of baro-tolerant variants of many microorganisms.

Abstract of 21 March 1980 - The work of the past year has focused on two main topics: the characterization of microbial growth inhibition by compressed gases and liquid anesthetics, and the effects of hydrostatic pressure on microorganisms. The work has involved both prokaryotic and eukaryotic microorganisms, and for the first time, we have started to use mammalian, tissue-culture cells.

We had previously concluded that growth modification by compressed gases and other anesthetics is not due to narcotic action but to a definably different class of actions. The potentiating action of helium for growth inhibition by nitrous oxide or oxygen was an important factor supporting our conclusion since helium is generally found to be anti-narcotic. The results of experiments carried out this year confirmed past findings of potentiation of growth inhibition by He, N₂ and Ar for bacteria, yeast and protozoa. None of these gases alone is inhibitory for growth, but each potentiates inhibition caused by nitrous oxide or oxygen.

The results of experiments carried out this year have indicated clearly that helium pressure is not equivalent to hydrostatic pressure and that helium has specific biological effects. In fact, a specific inhibitory action of helium for an isolated enzyme, xanthine oxidase, was demonstrated. Hydrostatic pressure acts to reverse growth inhibition of Saccharomyces cerevisiae caused by nitrous oxide; in contrast, compressed helium acts to potentiate inhibition. Helium appears, moreover, to antagonize the action of hydrostatic pressure, and compressed helium is considerably less inhibitory for growth than is hydrostatic pressure, at the same pressure. Compressed nitrogen or argon also appear to be antagonistic to hydrostatic pressure.

Hydrostatic pressure was found to have little effect on oxygen toxicity for yeast or bacteria, whereas helium {or nitrogen or argon} were potentiating. Again, helium seems to have specific biological actions, and compressed helium should not be used as a convenient means to apply hydrostatic pressure to microbial cells.

During the year, we found also that the narcotic antagonists naloxone and levallorphan do not reverse the growth inhibition due to nitrous oxide or heptanol. In addition, it was found that hydrostatic pressure will not reverse growth inhibition due to halothane or methoxyflurane, even though it will reverse inhibition due to nitrous oxide or heptanol.

A study of the effects of temperature on growth inhibition by N₂O revealed an unexpected response pattern with maximal resistance of S. cerevisiae to N₂O at 24°C and lower resistance at higher or lower growth temperatures. The pattern obtained is similar to that for an enzymatic or physiological process rather than for a purely physical one such as N₂O dissolution in membrane lipid.

Some exploration was made of the relationship between oxygen sensitivity and sensitivity to nitrous oxide. It was found that paraquat enhances sensitivity of Escherichia coli to both oxygen and nitrous oxide. A survey of many microorganisms for relative sensitivities to the two gases produced a complex picture which we

are currently attempting to analyze.

During the past year, we have been able to define what seems to be the major basis for enhanced barotolerance of the APR-11 variant of Streptococcus faecalis 9790. This barotolerant variant was found to be tolerant to acid conditions as well as high pressure. The bacterium was then found to have an arginine dihydrolase system which is not repressed by glucose in the normal way. Therefore, it degrades glucose and arginine at the same time. The ammonia from arginolysis then acts to neutralize the acid from glycolysis. In essence, the organism produces its own buffer. It is also able to produce more ATP per unit of time than the parent strain, and pressure tolerance appears to be related to this enhanced production and to the decreased acidification of the environment in APR-11 cultures.

Abstract of 31 March 1981 - Our work in the past year has been directed to two related topics. One has to do with the effects of hydrostatic pressure on microbial growth and metabolism. The other is concerned with the effects of compressed gases and other narcotic agents on growth and differentiation of microorganisms and on growth of tissue-culture cells.

One of the major components of the hydrostatic pressure project was a parametric analysis of the interactions of pressure with other environmental factors affecting microbial growth. For this analysis, we chose a set of standard conditions, specifically 37°C, 1 atmosphere and tryptone-glucose-Marmite medium, determined rate and extent of growth under these conditions, and then determined the various other sets of parametric values at which these standard growth responses occur. We were then able to prepare plots, for example, in the pressure-temperature plane of these sets. Such a plot outlines a contour, which gives a visual impression of the interactions between pressure and temperature affecting growth. During the year, we carried out such analyses for growth of Streptococcus faecalis and Bacillus licheniformis.

The other major project in microbial barobiology was on the effects of pressure on prokaryotic differentiation, specifically on pressure induced germination of endospores of Bacillus megaterium ATCC 19213. We were able to prepare various salt forms of the spores by means of an acidification-neutralization procedure that results in essentially complete exchange of minerals but, amazingly, little or no loss in viability in the population. We found that the hydrogen form is extremely resistant to pressure induced germination. The K form was less resistant, followed in series by the Mn, Ca, Mg, Na and native forms. A pressure of 493 atmospheres

was sufficient to induce over 80 % germination in a population of native spores in 2 hours. In contrast, this same pressure induced no germination in a population of fully viable hydrogen spores even after 19 hours of germination.

Much of the effort in our work on the biological effects of compressed gases was focused on animal cells in tissue culture, specifically HeLa cells and RRP 104cl0 haploid, frog cells. The gases were inhibitory for growth of both cell types, and we were able to determine 50% inhibitory pressures of 90 atm for He, 60 atm for Ar and 3 atm for nitrous oxide for the HeLa cells. Our major task now is to find out if these animal cells respond to the gases in the same manner that microbial cells do. In past research, we have found that modification of microbial growth by compressed gases cannot be viewed as a narcotic response but is in a different class of responses.

This year, we continued our study of the variation with temperature change in the growth inhibitory potency of nitrous oxide, now with E. coli and Tetrahymena. As we reported last year for yeast, there appears to be for each organism a temperature of maximal resistance to the gas with greater sensitivity at either higher or lower temperatures. This pattern is different from that usually found for simple narcotic responses.

Also, we have completed work on potency determinations for a wide variety of narcotic agents as they affect microbial growth. The net conclusion is that the agents fall into a number of definable classes and that one cannot reasonably relate growth inhibition potency simply to some physicochemical parameter such as lipid solubility.

During the period between March and September of this year, we have completed the study of the effects of specific mineralization on spores of Bacillus megaterium ATCC 19213 and have prepared a manuscript which has been submitted to the Canadian Journal of Microbiology. The abstract of the manuscript follows. Reprints will be forwarded to ONR when they are available.

"Spores of Bacillus megaterium ATCC 19213 were subjected to a complete ion-exchange regimen, which included titration to a pH value of 2, heating at 60°C for up to 18 h, back titration with various base solutions to a pH value of 8 and heating again at 60°C. Spore populations maintained high levels of viability throughout this rigorous procedure, and the various salt forms prepared showed a wide range of sensitivities to the germinating effect of hydrostatic pressure. Native spores showed the expected germination response when subjected to pressures of 350 to

750 atm {1 atm = 101.325 kPa.} at 24°C. There was a threshold pressure for germination of some 350 atm, and the apparent activation volume for the process was calculated to be 188 ml/mole, indicating that these spores had about the same pressure sensitivity as those of Bacillus subtilis or Bacillus pumilus. The optimum pH for germination was about 8, and the optimum temperature was ca. 45°C. The hierarchy of resistance of the various salt forms tested to the germinating action of 493 atm pressure was: H > K > Ca, Mg, Na > native. The H form was particularly insensitive even to pressures as high as 1,020 atm but did germinate in response to chemical germinants. We concluded that the specific mineralization of bacterial endospores has major influence on pressure-induced germination, which can occur even in the absence of added salts. As expected, sensitivities to pressure could not be correlated with previously reported heat sensitivities, electrostasis or states of dehydration."

During the period, other new work on the toxic actions of nitrous oxide and oxygen was completed, and the first draft of a manuscript has been prepared. The abstract of the manuscript follows.

"We have found that paraquat {methyl viologen} can enhance the toxicity of both O₂ and N₂O for the bacterium Escherichia coli. Hydroxyl radical scavengers moderate the toxic action of N₂O, as well as of O₂, and it seems that free radicals may be involved. Xenon was found to act in a manner superficially similar to N₂O, and He, N₂ or Ar enhanced the actions of Xe and N₂O."

During the period, we also completed the first draft of a manuscript in which the view that growth inhibition by anesthetics is not a narcotic response is further developed. It is based on data presented previously in annual reports.

During this final support period, studies with tissue-culture cells had to be suspended because of the expense involved. Other funds are now being sought.

B. Index of Annual Reports Issued Under the Contract

1. Technical Report Number 4 - 31 December 1975
2. Technical Report Number 5 - 31 March 1977
3. Technical Report Number 6 - 31 March 1978
4. Technical Report Number 7 - 31 March 1979
5. Technical Report Number 8 - 31 March 1980
6. Technical Report Number 9 - 31 March 1981
7. Final Report - 13 November 1981

C. Index of Publications Issued Under the Contract

1. Thom, S. R. and R. E. Marquis. 1981. Contrasting actions of hydrostatic pressure and helium pressure on growth of Saccharomyces cerevisiae. In: A. J. Bachrach and M. M. Matzen {Ed.} Underwater Physiology. Undersea Medical Society, Bethesda, Maryland. pp. 667-673.
2. Marquis, R. E. and G. R. Bender. 1980. Isolation of a variant of Streptococcus faecalis with enhanced barotolerance. Can. J. Microbiol. 26:371-376.
3. Marquis, R. E., S. R. Thom and C. A. Crookshank. 1978. Interactions of helium, oxygen and nitrous oxide affecting bacterial growth. Undersea Biomedical Res. 5:189-198.
4. Marquis, R. E. and P. Matsumura. 1978. Microbial life under pressure. In: D. J. Kushner {Ed.} Microbial Life in Extreme Environments. Academic Press, London. pp. 105-158.
5. Matsumura, P. and R. E. Marquis. 1977. Energetics of streptococcal growth inhibition by hydrostatic pressure. Appl. Environ. Microbiol. 33:885-892.
6. Marquis, R. E. 1976. High pressure microbial physiology. Adv. Microbial Physiol. 14:159-241.
7. Marquis, R. E. and D. M. Keller. 1975. Enzymatic adaptation by bacteria under pressure. J. Bacteriol. 122:575-584.

In process

1. Marquis, R. E. Microbial Barobiology, BioScience, in press.
2. Bender, G. R. and R. E. Marquis. Sensitivity of various salt forms of Bacillus megaterium spores to the germinating action of hydrostatic pressure. Submitted to Can. J. Microbiol.

D. Conclusions Drawn on the Basis of the Results

1. Growth inhibition by narcotic gases is not a narcotic response but belongs to a new and different class of responses.
2. The range of potencies of various types of anesthetic agents for microbial growth inhibition is not interpretable in terms of any simple physicochemical parameters such as lipid solubility.
3. The growth inhibitory actions of anesthetic gases apply to both prokaryotic and eukaryotic cells, including mammalian tissue-culture cells.

4. The ecological importance of hydrostatic pressure in the marine environment is much more extensive than previously considered - pressure can be a major ecological factor even in shallow water when otherwise nonoptimal growth conditions render organisms hypersensitive to pressure.

5. Growth of microorganisms can be optimized with respect to pressure in the same general way that one optimizes growth in respect to temperature and pH - 1 atm is not the optimal growth pressure for most bacteria when all environmental parameters are adjusted to give maximal growth.

6. Pressure-induced germination of bacterial endospores is very much affected by the specific mineralization of the cells.

7. Pressure-tolerant variants of ordinary laboratory bacteria can be isolated by use of pressure as a selective agent. However, care must be taken in selection to minimize cell damage resulting from increased sensitivities to acid conditions under pressure.

E. A List of Major Accomplishments

1. During the period of this contract, our laboratory has become one of the leading laboratories in the world for study of the barophysiology of microbes. Reviews of our work have been presented at a number of major, international conferences, including the conference on biotechnology held in Vancouver last August as part of the 1981 meeting of the International Union of Pure and Applied Chemistry and the Second International Symposium on Microbial Ecology held in Warwick, England, in September of 1980. The principal investigator has presented reviews at a number of major universities and has acted as a consultant on pressure problems in deep oil wells to the U. S. Department of Energy. We feel that the development of the laboratory is a major accomplishment of the contract and that it will remain a resource for our own future projects in microbial barobiology and a source of help to others undertaking related projects.

2. The uncovering of the complex nature of growth inhibition by narcotic gases and other anesthetic agents seems a major scientific advance with far-reaching application in basic cell physiology and in the more practical aspects of hyperbaric medicine, including cancer chemotherapy. There is a great deal of basic work to be done before these agents can be used medically to modify cell growth but the base has been set firmly.

3. Major accomplishments have been made also in the study of the responses of microbes to hydrostatic pressure. The isolation for the first time of a pressure-

tolerant variant of a common laboratory bacterium is noteworthy. We now have a much clearer view of the interaction of hydrostatic pressure with other environmental parameters affecting microbial growth and other functions, of the biological effects of low to moderate pressures, the effects of pressure on membrane functions, and the nature of spore germination induced by pressure. There is now a great deal of interest in extending these physiological findings to the molecular level so that an appreciation can be developed for the very basic aspects of biological responses to high pressure.

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4. The ecological importance of hydrostatic pressure in the marine environment is much more extensive than previously considered - pressure can be a major ecological factor even in shallow water when otherwise nonoptimal growth conditions render organisms hypersensitive to pressure.
5. Growth of microorganisms can be optimized with respect to pressure in the general way that one optimizes growth with respect to temperature and pH - 1 atm is not the optimal growth pressure for most bacteria when all environmental parameters are adjusted to give maximal growth.
6. Pressure-induced germination of bacterial endospores is very much affected by the specific mineralization of the cells.
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